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TGFβ1 rapidly activates Src through a non-canonical redox signaling mechanism

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Abstract

Transforming growth factor-β1 (TGF-β) is involved in multiple cellular processes through Src activation. In the canonical pathway, Src activation is initiated by pTyr530 dephosphorylation followed by a conformational change allowing Tyr419 auto-phosphorylation. A non-canonical pathway in which oxidation of cysteine allows bypassing of pTyr530 dephosphorylation has been reported. Here, we examined how TGF-β activates Src in H358 cells, a small cell lung carcinoma cell line. TGF-β increased Src Tyr419 phosphorylation, but surprisingly, Tyr530 phosphorylation was increased rather than decreased. Vanadate, a protein tyrosine phosphatase inhibitor, stimulated Src activation itself, but rather than inhibiting Src activation by TGF-β, activation by vanadate was additive with TGF-β showing that pTyr530 dephosphorylation was not required. Thus, the involvement of the non-canonical oxidative activation was suspected. TGF-β increased extracellular H_2O_2 transiently while GSH-ester and catalase abrogated Src activation by TGF-β. Apocynin, a NADPH oxidase inhibitor, inhibited TGF-β-stimulated H_2O_2 production. Furthermore, mutation of cysteines to alanine, 248C/A, 277C/A, or 501C/A abrogated, while 490C/A significantly reduced, TGF-β-mediated Src activation. Taken together, the results indicate that TGF-β-mediated Src activation operates largely through a redox dependent mechanism, resulting from enhanced H_2O_2 production through an NADPH oxidase and that cysteines 248, 277, 490, and 501 are critical for this activation.

Keywords

Src; TGF-β; hydrogen peroxide; oxidative modification; redox signaling

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Introduction

Src is a ubiquitously expressed non-receptor tyrosine kinase belonging to the Src family of protein tyrosine kinases (SFKs). By coupling signals from cell surface receptors and various intracellular signaling pathways (1), Src is involved in various fundamental cellular processes, including proliferation, differentiation, and transformation (2). Src also plays a critical role in epithelial-mesenchymal transition (EMT) (3-7), a process implicated in both wound healing and cancer metastasis (8-11).

In the canonical Src activation pathway, under basal conditions *in vivo*, a Src molecule remains inactive as a result of the intramolecular interactions between its SH3 domain and linker region, and its SH2 domain and the phosphorylated Tyr530 (pTyr530) in the C-terminal negative regulatory region (12,13). Upon stimulation, pTyr530 is dephosphorylated thus dissociating the inhibitory intracellular interactions and allowing Src to autophosphorylate at Tyr419 and restoring full activity (14-17). Thus, in the canonical pathway phosphorylation/dephosphorylation of Tyr530 plays a critical role in Src activation/deactivation. Several kinases and protein tyrosine phosphatases (PTPs) are involved in Src regulation (18), including C-terminal Src kinase (Csk) and Csk homologous kinase (Chk), that phosphorylate Src at Tyr530, and PTP1B, SHP1, PTPα, or PTPγ that reportedly catalyze Tyr530 dephosphorylation (18).

It has been well established that reactive electrophiles, such as H_2O_2, can participate in signal transduction by acting as second messengers through modification of the activity of signaling molecules. This so called redox signaling mechanism underlies the regulation of signaling pathways induced by many stimuli, including various physiological and/or pathological stimuli. Signaling molecules containing redox sensitive moieties such as cysteine, which readily reacts with electrophiles, have been found to be potential targets of regulation through redox mechanisms. PTPs have been recognized as major switches in redox signaling (19-25). PTPs all have a catalytic site structure in which a reactive cysteine moiety (26,27) is potentially susceptible to oxidative modification by H_2O_2. The oxidization is however, likely mediated by an enzyme, as the non-enzymatic rate of reaction with H_2O_2 is too slow to account for the inactivation (28). Redox modification inhibits the activity of PTPs and leads to increased phosphorylation of their corresponding substrate signaling molecules, resulting in altered signaling pathways.

Accumulating evidence suggests that Src can also be activated through a redox dependent mechanism. Purified Src is activated *in vitro* through either oxidation or alkylation of two cysteine (cys) residues on the protein (29). *In vivo*, Src is activated by various reactive oxidants such as cigarette smoke (30), acrolein (30), peroxynitrite, and hydrogen peroxide (H_2O_2) (31-34). In addition, Src activation by many other stimuli including some growth factors seems to involve redox mechanisms (35-38).

There are nine cysteine residues in human Src that are highly conserved among Src family members and species (39), some of which have been implicated in Src activity regulation by oxidative stimuli. Senga first reported that mutation of Cys487 in v-Src (corresponding to Cys 490 in human Src) suppressed its activity while mutation of others changed Src stability

(39). In an *in vitro* assay, Cys277 seemed critical for the homodimerization and activation of Src (40). Giannoni *et al.* found that Cys245 and Cys487 in v-Src (corresponding to Cys248 and Cys490 in human respectively) were involved in Src activation by oxidants, and dephosphorylation of Tyr530 was important for the early (10min) activation of Src, while a redox mechanism was involved in the late phase of Src activation (45min) in response to extracellular matrix (35).

Transforming growth factor-β1 (TGF-β) is a potent multifunctional growth factor involved in the regulation of cellular proliferation, differentiation and survival, and plays a predominant role in the EMT process (41). TGF-β initiates signaling through binding to its type II receptor, which recruits and phosphorylates type I TGF-β receptor. The type I receptor, a serine/threonine protein kinase, phosphorylates and activates diverse downstream signaling pathways, including ERK, JNK, p38MAPK, PI3K/AKT, and transcription factors such as SMAD2/3 (42).

Many effects of TGF-β are mediated through Src-mediated signaling pathways (43-45). How TGF-β activates Src is not completely clear. Some studies demonstrated that TGF-β could induce the production of oxidants that contributed to TGF-β-mediated effects (43,46), however, little is known about the role of oxidants in the activation of Src by TGF-β. Here, we report that a redox dependent mechanism, most likely involving cysteines of both Src and the PTPs that regulate its activity, is involved in Src activation by TGF-β in a non-canonical redox activation of Src.

Materials and Methods

Chemicals and reagents

Unless otherwise noted, all chemicals were from Sigma (St. Louis, MO). Antibodies to Src and phosphorylated Src were from Cell Signaling Technology, Inc. (Danvers, MA). M-PER Mammalian Protein Extraction Reagent was from Thermal Fisher Scientific Inc. (Thermal Fisher, Rockford, IL). Amplex Red reagent was from Life Technologies (Grand Island, NY). All chemicals used were at least analytical grade.

Cell culture and treatment

A human non-small cell lung carcinoma cell line (H358) was used. H358 cells were cultured in RPMI-1640 medium with 10% fatal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin, in a humidified incubator containing 5% CO_2 at 37°C. Cells were treated when about 85% confluent. In catalase experiment, catalase (final concentration is 30 U/ml) was added to the culture medium immediately before TGF-β exposure.

Western Analysis

Briefly, cell lysate was extracted with M-PER and 30 μg protein was electrophoresed on a 4-20% Tris-glycine acrylamide gel (Invitrogen, Carlsbad, CA) and transferred to polyvinylidene difluoride (PVDF) membranes (Millipore Corporation, Bedford, MA). Membranes were blocked with 5% fat-free milk and then incubated overnight at 4°C with primary antibody in 5% BSA dissolved in Tris-buffered saline (TBS). After being washed

with 1XTBS containing 0.05% Tween 20 (TTBS), membranes were incubated with secondary antibody at room temperature for 2 h. After TTBS washing, membranes were treated with an enhanced chemiluminescence reagent mixture (Thermal Fisher Scientific, Rockford, IL) for 5 min and then imaged and analyzed using the Biospectrum imaging system (UVP, Upland, CA)

Immunoprecipitation

Cells were washed with cold phosphate-buffered saline (PBS) and collected in 1 ml lysis buffer containing 50 mM HEPES (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.5% NP40, 50 mM β-glycerophosphate, 1 mM sodium orthovanadate, 10 mM sodium pyrophosphate, 1 mM EGTA, 1 mM PMSF, 10% glycerol, 100 mM sodium fluoride, 10 μM DETAPAC, 10 ng/ml leupeptin, and 10 ng/ml aprotinin. 500 μg of whole cell lysate was used for immunoprecipitation with ANTI-FLAG M2 Affinity Gel (Sigma-Aldrich) overnight at 4°C. The complexes were washed three times with TBS, re-suspended in 100 μl of TBS containing 3 μg of 3XFLAG peptide, and incubated at 4°C for 30 min with shaking. After centrifuge, the supernatant was collected and used for Western Blots.

Construction of FLAG-Tagged Src plasmids, site-directed mutagenesis, and transfection

pcDNA3 c-Src (47) (Addgene plasmid 42202) was digested with Hind III and Xba I to get 1.6kb cDNA of human c-Src. The 1.6kb c-Src cDNA was then inserted into p3XFLAG-CMV-8 Expression vector (Sigma). The plasmid was selected with ampicillin and confirmed with DNA sequencing.

Site directed mutagenesis was then performed as described before (48) using a kit (GeneArt Site Directed Mutagenesis System, Invitrogen). The primer sequences were as following (only forward primer was shown): Src248C/A, CCTCACCACCGTGTTCCCCACGTCCAAG; Src277C/A, GCTGGGCCAGGGCTTCTTTGGCGAGGTG; Src490C/A, CTGCCCGCCGGAGTTTCCCGAGTCCCTG; Src501C/A, CCTCATGTGCCAGTTCTGGCGGAAGGAG. The mutations were confirmed with DNA sequencing.

Cells were transfected with plasmids with Lipofectamine 2000 when 90% confluence. Briefly DNA and Lipofectamine were mixed at 1:3.5 ratio and incubated at RT for 15 min before being added to cells. The medium was replaced the next day and 48 h after transfection, cells were treated and collected for experiments.

H_2O_2 measurement

H_2O_2 was measured in the extracellular media using Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine). Before treated with TGF-β cell medium was replaced with Krebs–Ringer phosphate glucose (KRPG) (145 mM NaCl, 5.7 mM sodium phosphate, 4.86 mM KCl, 0.54 mM $CaCl_2$, 1.22 mM $MgSO_4$, 5.5 mM glucose, pH 7.35). 120 μl of KRPG was collected at different time points after adding of TGF-β. To measure H_2O_2, 50 μl of sample was mixed with same volume of reaction buffer containing Amplex red and horseradish peroxidase. The fluorescence signal was detected kinetically with excitation wavelength at

529 nm and emission wavelength at 590 nm. The H_2O_2 concentration was then calculated based on standard curve.

Statistical analysis

Data were expressed as mean ± standard error. Wilcoxon rank-Sign test was used for statistical analysis of Western densitometry data and t-test was used for analysis of H_2O_2 data. Statistical significance was accepted when $p < 0.05$.

Results

TGF-β activated Src in redox-dependent manner

In the canonical pathway, Src activation depends on dephosphorylation of pTyr530 and subsequent phosphorylation at Tyr419. To determine whether Src activation by TGF-β follows this mechanism, we first examined the phosphorylation status of Src at Tyr419 and Tyr530 at different times after TGF-β exposure. As shown in Figure 1, after addition of TGF-β (5 ng/ml), phosphorylation of Src at Tyr419 (pTyr419Src) began to increase at 15 min and reached its highest level at 30 min. Src activation was sustained until it declined to control level after 2 hours of TGF-β exposure. The phosphorylation of Src at Tyr530 (pTyr530Src) followed a similar pattern; it increased at 15 min, reached its highest level at about 30 min, and decreased to control level after 2 hour.

Effect of vanadate on Src activation by TGF-β

This increase in both pTyr419Src and pTyr530Src phosphorylation upon TGF-β stimulation demonstrated that dephosphorylation of pTyr530 was not essential for TGF-β-mediated Src activation. To further examine this deviation from the canonical pathway, cells were pretreated with sodium orthovanadate (vanadate) for 30 min before being exposed to TGF-β. Vanadate is a general PTP inhibitor and would be expected to block the enzymatic dephosphorylation of Src at Tyr530. Interestingly, vanadate increased both basal and TGF-β-mediated pTyr530 Src (Figure 2). This suggests that both the tyrosine kinase that catalyzes phosphorylation of Tyr530 and the PTP that dephosphorylates Tyr530 are endogenously active.

The effect of vanadate on the TGFβ-stimulated phosphorylation of Tyr419Src had a more complex dose-dependency. At 0.5 mM, vanadate appeared to inhibit both endogenous Src activation and Src activation by TGFβ. At 2 mM vanadate alone activated Src and appeared additive with TGFβ in activating Src. At 10 mM vanadate alone increased the level of pTyr419Src further and abrogated further stimulation of Src by TGF-β. As Src activation by vanadate was additive with TGF-β, pTyr530 dephosphorylation was clearly not required for Src activation by TGF-β. The results are possibly due to differential sensitivity to vanadate inhibition of PTPs involved in the dephosphorylation of pTyr530 and pTyr419. It appears that Tyr530 dephosphorylation is more sensitive to vanadate, resulting in greater pTyr530 at lower vanadate concentrations, and leading to more Src in the inhibitory form of pTyr530 and less pTyr419. At higher vanadate concentrations, both the dephosphorylation of pTyr530 and pTyr419 were inhibited, and this resulted in increased pTyr530 and pTyr419.

Induction of extracellular H_2O_2 by TGF-β

Previous studies have shown that a redox mechanism is implicated in many pathophysiological functions of TGF-β (43,46,49). As Src is a critical mediator of TGF-β signaling and its activity is regulated by oxidants. Thus, we hypothesized that Src activation by TGF-β could be redox-dependent. To test this, we first determined the production of H_2O_2 by TGF-β using Amplex Red. Upon TGF-β exposure (5 ng/ml), extracellular H_2O_2 was significantly increased at as early as 1 min, reached its highest level at 10 min, and was back to basal level in 20 min (Figure 3). Catalase (30 U/ml), which catalyzes the dismutation of H_2O_2 to O_2 and H_2O, completely removed the basal and TGF-β-produced H_2O_2 in the extracellular environment. H_2O_2 was produced continuously by H358 cells (Figure 3). Media incubated without cells did not produce any H_2O_2.

Redox dependent Src activation by TGF-β

To further study the redox-dependence of Src activation by TGF-β, we next examined the effect of antioxidants. GSH-ester, a precursor of intracellular GSH, or catalase, which removed extracellular H_2O_2 as shown above, blocked Src activation (pTyr419Src) by TGF-β (Figure 4), indicating that TGF-β-mediated Src activation is accomplished through a redox dependent mechanism. Nonetheless, pretreatment with GSH-ester or catalase increased basal Src activation as shown by a significant elevation of basal pTyr419Src (Figure 4). This occurred concurrently with a decrease in pTyr530Src, suggesting that the basal level of Src activation was through redox dependent dephosphorylation of pTyr530. One possible explanation for this phenomenon could be the relief by GSH-ester or catalase of the inhibition of a PTP, as such enzymes are well known targets for inhibition by H_2O_2 (26,27).

Involvement of NOX in TGF-β-mediated H_2O_2 production and Src activation

To determine the source of H_2O_2, cells were pretreated with apocynin, an inhibitor of NADPH oxidases (NOX), before being treated with TGF-β. Pretreatment with 300 μM apocynin for 30 min abrogated the increase of H_2O_2 caused by TGF-β, suggesting that TGF-β produced H_2O_2 through activating NOX (Figure 5).

Mutation of cysteine residues decreased Src activation by TGF-β

Cysteine residues in Src are the most likely targets of oxidative modification resulting in Src regulation. To identify the cysteine residue(s) in human Src involved in Src activation by TGF-β, cysteine residues at 248, 277, 490, or 501 were replaced individually with alanine, using site-directed mutation. These cysteine residues were selected because of their conservation among Src kinase family members across species (39), or their reported sensitivity to redox modification (35,40,50). As shown in Figure 6, the phosphorylation of Src at Tyr419 and Tyr530 were affected in different ways by the cysteine/alanine mutation. The 248C/A had no effect on the basal level of pTyr419 or pTyr530 of Src. The 277C/A mutation did not affect the basal pTyr530Src, but decreased the basal level of pTyr419 Src by 50%. The 490C/A mutation decreased the basal level of pTyr530 by 67% and that of pTyr419 by 50%, while the 501C/A mutation decreased the basal level of pTyr530 and pTyr419 both by about 80%. TGF-β-mediated Src activation was abrogated by C/A

mutation at residues 248, 277, or 501, while it was decreased significantly in 490C/A Src (52% vs. 120% induction in 490C/A and wild type FLAG-Src respectively).

Discussion

Src activation is responsible for many TGF-β-mediated effects (43-45). The mechanism underlying Src activation by TGF-β however, has been unclear. This study demonstrates that Src activation by TGF-β is largely through a redox-dependent pathway. In addition, we verified that some cysteine residues in Src are critical for Src regulation and mutation of cysteine at 277, 490 and 501 to alanine decreases the basal Src activity while mutation of cysteine residues at 248, 277, and 501 abrogates TGF-β-mediated Src activation.

In the canonical pathway, Src activation is initiated by dephosphorylation of pTyr530 followed by a conformational change and subsequent dissociation of intracellular interactions and autophosphorylation of Src at Tyr419 (Figure 7B). Our data (Figure 1) suggest that a non-canonical mechanism, which does not require the dephosphorylation of pTyr530, was involved in TGF-β-mediated Src activation. Inhibition of PTPs (by vanadate at 0.5 and 2 mM concentration), which are responsible for the dephosphorylation of pTyr530, increased pTyr530 Src, but only inhibited TGF-β-mediated Src activation by~40%. In other words, ~60% of Src activation was independent of the dephosphorylation of pTyr530. This evidence further supports that both canonical and non-canonical pathways are involved in TGF-β-mediated Src activation.

The finding that antioxidants (GSH-ester and catalase) inhibited TGF-β-mediated Src activation indicated that Src activation by TGF-β operated, at least partially, through a redox-dependent mechanism. The results demonstrated a more complex relationship between oxidative Src activation and phosphorylation/dephosphorylation of Src tyrosines. Somewhat surprisingly, antioxidants alone decrease the phosphorylation of Src at Tyr530 while increasing pTyr419. Indeed, this suggests that in H358 cells, there is the potential for endogenous activation of Src through the canonical pathway that is inhibited by the endogenous H_2O_2. Removing H_2O_2 then reveals this endogenous Src activation. Most likely, the PTPs that dephosphorylate pTyr530 are inhibited by the endogenous H_2O_2 so that preventing its inactivation allows dephosphorylation of pTyr530 and subsequent autophosphorylation of Tyr419; i.e., the canonical pathway. In contrast, Src activation by TGF-β was, at least partially, independent of the dephosphorylation of pTyr530. Consistent with our finding, Akhand also found that nitric oxide-mediated Src activation was independent of the phosphorylation status of Tyr530 (51,52). This was further supported by a study from Pu et al. that Hg^{2+} activated Src in a redox dependent way with the mutation of Tyr530 or inhibition of its phosphorylation (51,52). The PTPs involved in the dephosphorylation of pTyr530, such as PTP1B (26,27) and SHP-1 (53) are redox sensitive. At resting condition, under which H_2O_2 is constantly produced, a small portion of the PTPs are inhibited. The addition of antioxidants would remove this basal H_2O_2 and thus reduce the inhibition on PTPs, resulting in increased dephosphorylation of pTyr530 and a subsequent increase in Src activity (the canonical pathway of Src activation).

Except for PTPs, kinases that phosphorylate Tyr530, including CSK and CHK, can also be regulated through redox-dependent mechanisms. Mills *et al.* reported that CSK activity could be regulated through the oxidation state of the disulfide bond in the SH2 domain, implying that CSK activity can be affected by redox changes in cells (54). H_2O_2 production may facilitate disulfide bond formation and increase CSK activity, leading to increased pTyr530. Furthermore, CSK activity can also be negatively regulated by Src through its phosphorylation of the CSK binding protein (CBP), which recruits CSK to the membrane to which Src translocates (55). Therefore the phosphorylation status of Tyr530 is regulated by a complex network with the net activity determined by the overall effect of these pathways. In contrast to current findings, Parasassi reported that supplementation with antioxidants such as N-acetylcysteine and dithiothreitol could decrease Src activity by up to 30% in a manner independent of the phosphorylation status of Src530 (56). This inconsistency may be related to differences in kinases and PTPs involved in the regulation of the phosphorylation of either Tyr530 or Tyr419 in the cell types used. Nonetheless, our data suggest that the basal Src activity is regulated through redox-dependent dephosphorylation of pTyr530, while the TGF-β-mediated Src activation operates mainly through a redox-dependent mechanism.

Previous studies have found that TGF-β generates H_2O_2 through several sources including mitochondria (57), NOX1, NOX2 (58), and NOX4 (59). Here, we measured H_2O_2 in the extracellular medium where H_2O_2 is potentially produced by a membrane NADPH oxidase or through the plasma membrane following cytosolic production. The appearance of induced extracellular H_2O_2 in the current study was observed significantly earlier than in previous reports of TGF-β-induced intracellular production of H_2O_2, which occurred 30 min after TGF-β exposure and was purportedly due to mitochondria (57) or after 8 h by the intracellular NOX4 (59). Here, TGF-β appears to generate H_2O_2 through a NOX, as the NOX inhibitor apocynin blocked elevation of H_2O_2 and Src activation caused by TGF-β (Figure 5). The inhibitory effect of apocynin on NOX depends upon the formation of diapocynin, the dimer form, through a process that is cell-type dependent (60). As it is unclear whether diapocynin is formed in H358 cells, we cannot be certain of the mechanism of the inhibition of apocynin of TGF-β-induced H_2O_2 production. While H_2O_2 from NOX4, the only NOX located in the cytosol, may affect Src activity later and modulate later events, the early production of H_2O_2 and activation of Src observed here implies involvement of a plasma membrane associated NOX rather than NOX4. The evidence supporting this assertion is that the inhibition of TGF-β-induced Src activation by extracellular catalase would not affect intracellular production of H_2O_2 because the large extracellular/intracellular volume would dilute H_2O_2 coming from an intracellular source even in the absence of catalase. Instead, the likely source is a membrane oxidase that increases H_2O_2 production extracellularly, either directly or through superoxide production and subsequent dismutation to H_2O_2 and O_2.

A possible source of TGF-β produced extracellular H_2O_2 is NOX1 or NOX2. Rac and $p47^{phox}$, components of active NOX1 and 2 complex, are potential downstream target of TGF-β signaling (58,61). Another potential source of H_2O_2 is DUOX1, a predominant membrane NADPH oxidase in bronchial epithelial cells (62). DUOX1 can be activated

through association with $p47^{phox}$ as a result of lipid raft clustering caused by stimulators including TNFα (63). Indeed, TGF-β was found to cause lipid raft location of TGF-β receptors and regulate signal transduction (64-66). Which member of the NOX family is involved has not been determined, but is beyond the scope of the present study.

The redox dependence of TGF-β-mediated Src activation is further supported by the finding that activation was abrogated by mutation of cysteine residues in Src. Mutation of Cys248, Cys277, or Cys501 to alanine completely inhibited, while that of Cys490 partially inhibited TGF-β-mediated Src activation; these findings suggest that cysteines 248, 277. 501, and 490 might be the targets of oxidative modification by H_2O_2 produced by TGF-β. Our results are consistent with those of Giannoni, *et al.*, who found that Cys245 and Cys498 in v-Src (equal to Cys248 and Cys501 respectively in human Src) were critical for Src activation in response to ECM (35). Mutation of 490C/A and 501C/A decreased the basal level of pTyr419 and pTyr530 significantly, indicating that mutation of these two cysteines to alanines might cause conformational change of Src that result in inaccessibility of these tyrosine sites to kinases. Senga, *et al.* also reported that mutation of Cys498 of v-Src could suppress Src transforming activity (39). However, it remains to be further clarified how these C/A mutations affect Src regulation.

Among the 9 cysteine residues in human Src kinase, Cys 188, 241, and 248 are in the SH2 domain; Cys 277 and 403 are in the linker region; and Cys486, 490, 499 and 501 are in the kinase domain (Figure 7A). The current study and others (35), support the concept that Cys248, 277, 490, and 501 play critical roles in the regulation of Src activity. Indeed, mutation of cysteine residues in the C-terminus (such as Cys490 and 501) makes Src insensitive to redox regulation (29). On the other hand, there are also reports that some cysteine residues in Src might be involved in the suppression of Src activity. For instance, Cys277 was found to be responsible for oxidant-mediated Src homodimerization and inactivation in an *in vitro* assay (40), and mutation of Cys403 and Cys486 increased Src activity (50). Our data support that cysteine residue 248, 277, 490 and 501 of human Src are essential for Src activation by TGF-β and are potential targets of oxidative modification. Direct modification of cysteine residues in Src has been observed during activation by oxidative stimuli such as NO, herbimycin, and other agents (31,51,67). Interestingly Byeon, et al. demonstrated that a hydroquinone could bind to cysteine residues and cause Src activation (50). This evidence allows for the possibility that some cysteine residues in Src might be direct targets of oxidative modification. It still remains largely unknown, however, how cysteine modification actually causes Src activation. Some studies suggest there are intramolecular disulfide bonds that inhibit Src activity, and that cysteine modification could disrupt these disulfide bonds and activate Src (50). Other studies suggest that disulfide formation activates Src (14). Our current studies show that cysteine mutation to alanine, which prevents formation of disulfide bonds, is more consistent with the latter study.

Conclusion

In summary our data demonstrate three aspects of Src activation by TGF-β in H358 cells. H_2O_2 generation inhibits Src activation, apparently through inhibition of the PTPs that dephosphorylate pTyr530. In surprising contrast, TGF-β causes Src activation primarily

through a redox-dependent, non-canonical pathway that is independent of the dephosphorylation of pTyr530. Finally, mutation of conserved cysteines inhibits TGF-β induced Src activation possibly by preventing disulfide formation.

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Highlights

1. TGF-β activates Src in H358 cells largely through a non-canonical redox mechanism.
2. TGF-β induces a transient increase in extracellular H_2O_2 with a peak at 10 min.
3. Cysteine mutations in Src abrogate Src activation by TGF-β.

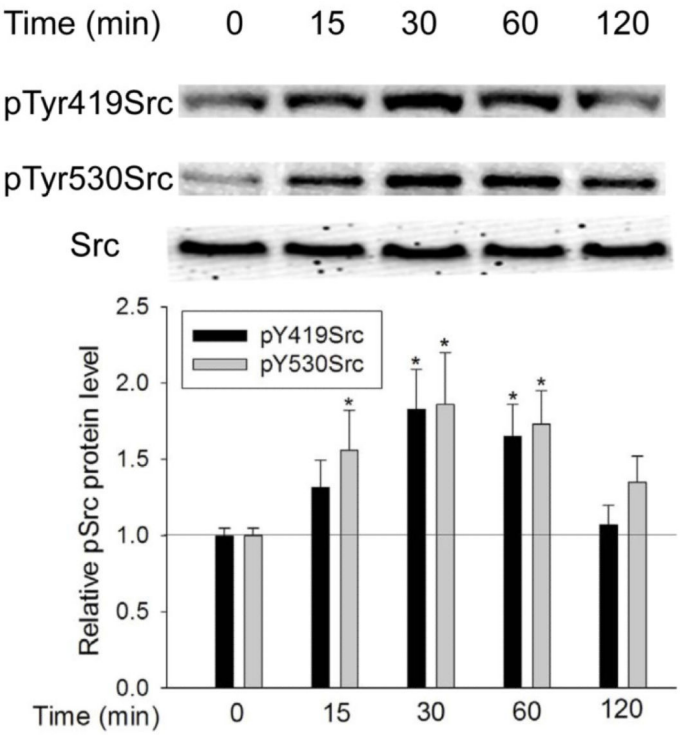

Figure 1.

TGF-β increased Src phosphorylation at both Tyr419 and Tyr530. Cells were exposed to 5 ng/ml TGF-β for the indicated times and pTyr419 and pTyr530 Src were determined by Western Blotting. *, P<0.05, **, P<0.01, compared with control, N=3.

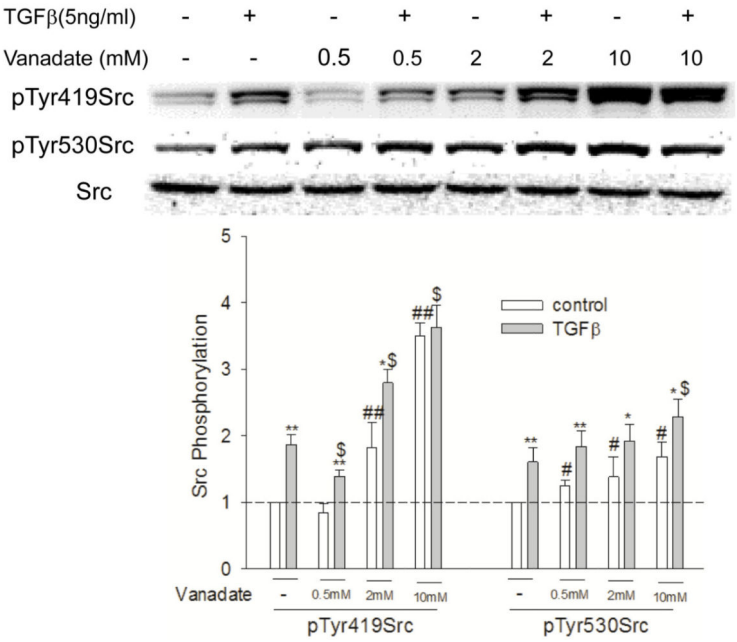

Figure 2.

Vanadate reveals non-canonical basal and TGF-β-stimulated Src activation. Cells were pretreated with different concentrations of vanadate for 1h before being exposed to TGF-β, the cells were then collected and Src phosphorylation at Tyr419 and Tyr530 was determined by Western Blotting. *, P<0.05, **, p<0.01, compared with vehicle control; #, p<0.05, ##, P<0.01, compared with control of no TGF-β and no vanadate exposure; $, P<0.05 compared with TGF-β treatment, N=3.

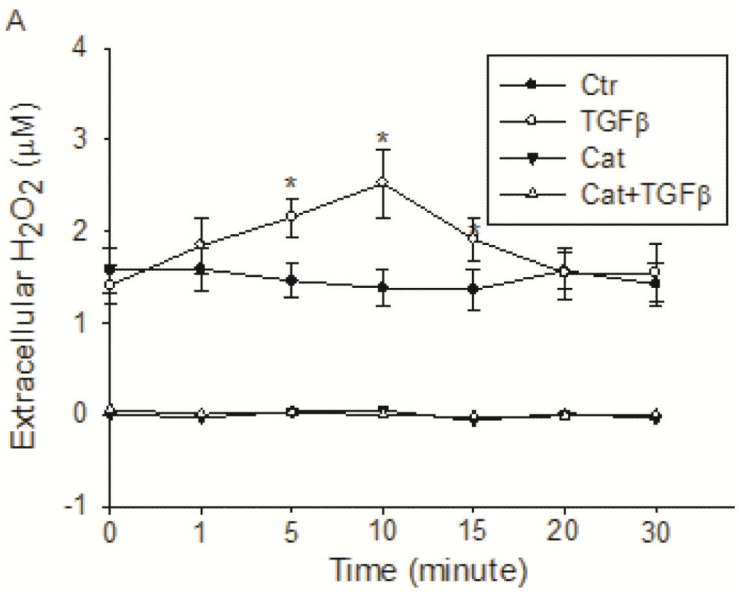

Figure 3.

TGF-β induced increase in extracellular H_2O_2. H358 cells were exposed to 5 ng/ml TGF-β for the times indicated and the extracellular H_2O_2 level was measured with Amplex Red reagent. *, P<0.01 compared with control, N=3.

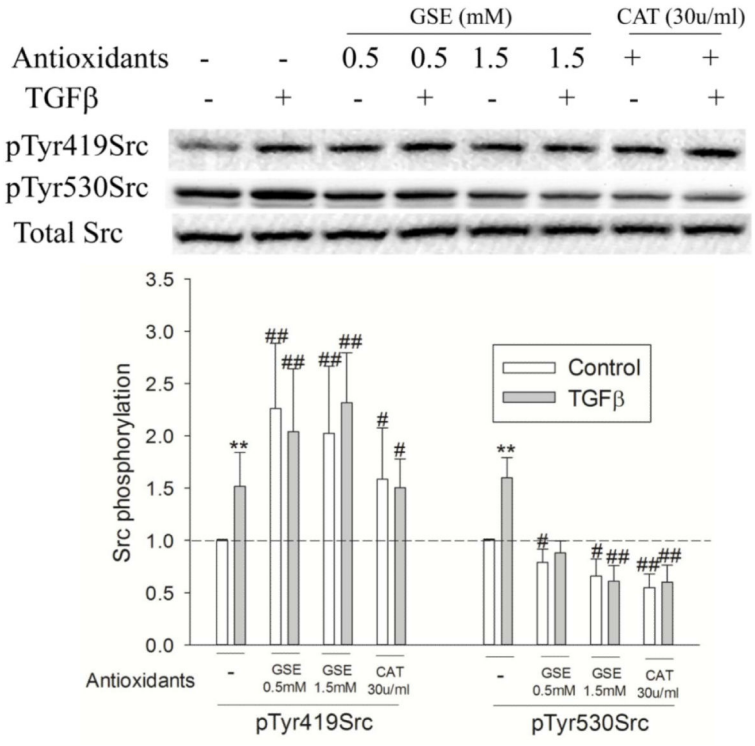

Figure 4.

Effect of antioxidants on TGF-β-mediated Src activation. Cells were exposed to 5 ng/ml TGF-β for the indicated times and pTyr419 and pTyr530 Src were determined by Western Blotting. *, P<0.05, **, P<0.01 compared with control; #, P<0.05, ##, P<0.01, compared with control of no TGF-β, N=3.

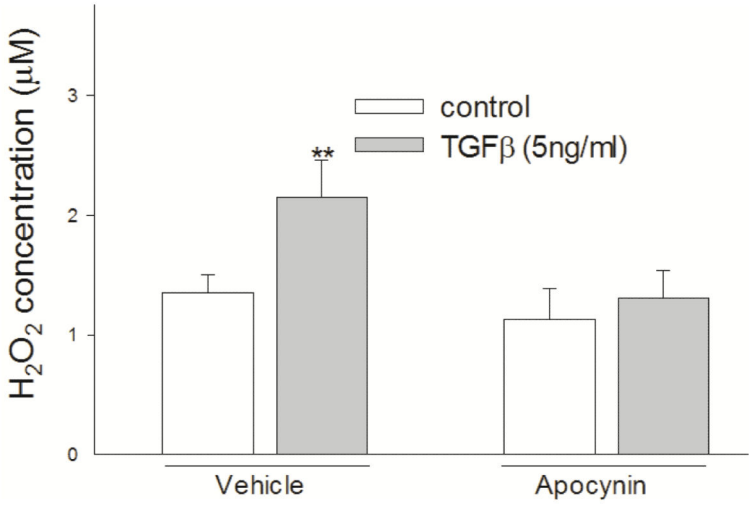

Figure 5.

TGF-β generated H_2O_2 through NOX. Apocynin abrogated TGF-β-caused increase of extracellular H_2O_2 level. H358 cells were pretreated with/without 300 μM apocynin for 30 min and then treated with 5 ng/ml TGF-β for 10 min. H_2O_2 was measured using Amplex Red method. **, P<0.01 compared with control, N=3.

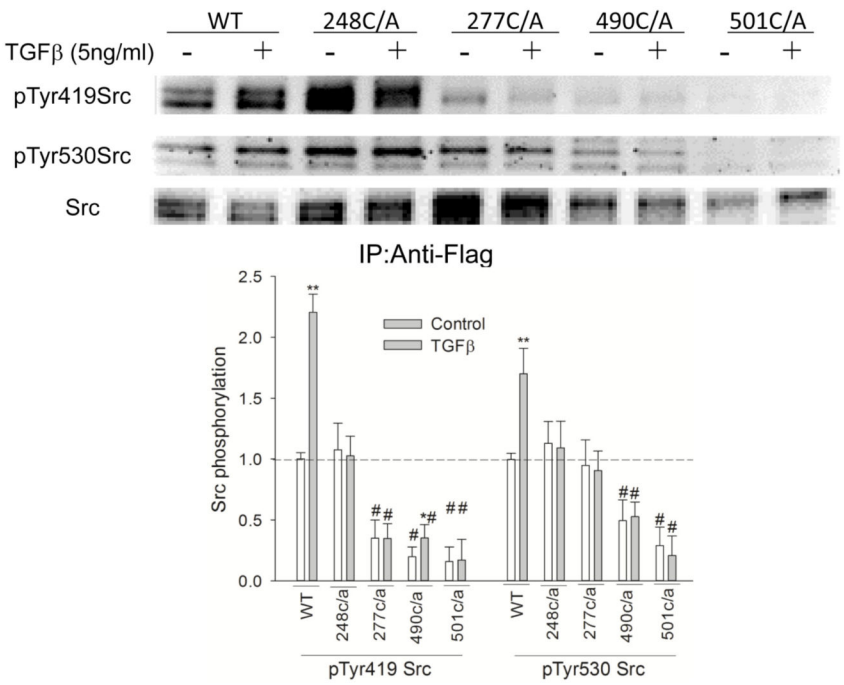

Figure 6.

Mutation of cysteine residues in Src decreased Src activation by TGF-β . H358 cells were transfected with FLAG-tagged Src plasmids with/without cysteine/alanine mutations for 24 h and then exposed to 5 ng/ml TGF-β for 1h. FLAG-Src was immunoprecipitated with Anti-FLAG agarose beads and then the phosphorylation of Src was determined by Western blotting. *, P<0.05, **, P<0.01 compared with control, #, P<0.01, compared with control of no TGF-β, N=3.

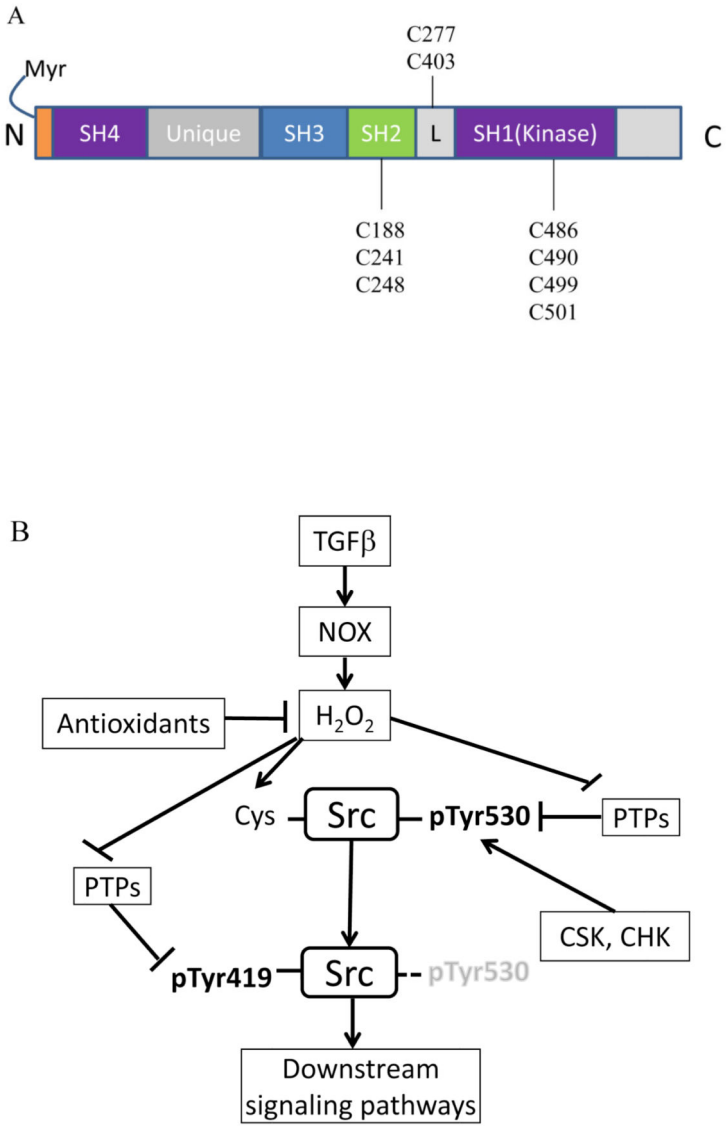

Figure 7.

Redox regulation of Src by TGF-β. (A) Location of cysteine residues in Src protein; (B) possible targets of redox regulation in Src activation pathway by TGF-β.